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A polymer-based DNA biochip platform for human papilloma virus genotyping

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A B S T R A C T

Genotyping of the human papilloma virus (HPV) is from a clinical point of view an important diagnostic task as some genotypes play a major role in the development of cervical carcinoma. So far PCR combined with blotting or *in situ* labelling is known to be the most accurate and sensitive method for detection and genotyping of HPV infection in clinical samples. However, specificity, cost-efficiency and sensitivity are not always satisfactory. A novel DNA biochip is described based on a plastic substrate, onto which small polymer droplets and single-stranded DNA are printed in the form of microarrays. Immobilisation of all compounds on the chip surface is achieved by a short UV-irradiation process, inducing photochemical reactions in the polymer. The chip designed for this study contains 36 probes for determining 12 common, different HPV genotypes. After isolation of the DNA, PCR and biochip read-out, the chip allows for genotyping of the most common virus strains, which, according to current prevalence studies, cover 85–95% of all infections. Following this approach as little as 10 virus copies can be detected within a short exposure time. Even using paraffin-embedded material and 10^4 copies per PCR are sufficient to allow rapid and reliable HPV genotyping.

1. Introduction

The Papanicolaou (PAP) smear test is one of the standard techniques for detecting the human papilloma virus (HPV) (Matthews-Greer et al., 2004). Because of the known lack of sensitivity of this test and problems both with false-negative and false-positive results, it is assumed that DNA testing might eventually replace cytology for routine gynaecological screening (Kuebler et al., 2007). For the prognosis of tumourigenic progression (mainly for cervical and laryngeal cancer), it is important that not only the incidence of an infection is verified, but also that the HPV genotype is known (zur Hausen, 2000, 2002). Within the last 2 years the first highly effective preventive vaccines (Menzo et al., 2007; Wu, 2007) were introduced. Since vaccination effectiveness needs to be verified, the commercial introduction of vaccines has increased the demand for early detection and determination of HPV infection, especially of the widespread HPV genotypes 16 and 18 (Villa, 2007).

A number of PCR-based kits for genotyping HPV are currently commercially available. Although PCR is known to be the most sensitive method for detection of HPV infection in clinical samples (Qu et al., 1997; Kleter et al., 1999), it also has some drawbacks. Firstly, there is an inherent danger of false-positive results due to non-specific amplification. Secondly, false-negative results may occur due to variations of the primer binding sites in the target region of the virus DNA, which in turn would lead to lower amplification signals of some HPV genotypes. Because of this problem, the PCR method may not detect all HPV genotypes present in the sample. Another downside is that multiple infections are not uncommon. Amplification of samples that contain DNA from more than one HPV genotype can lead to a much stronger amplification of one of the sequences present, which would complicate the detection of all HPV genotypes present. Sometimes additional labour-intense procedures, such as sequencing or type-specific PCR, are required (Wang et al., 2002). Recently, first studies involving genotyping of HPV with genotype-specific oligonucleotides and DNA microarray analysis have been reported (An et al., 2003, 2005; Hwang et al., 2003). However, these methods are still far from routine diagnostics as the costs of materials and labour are high.

In the past years there has been a remarkable progress in the development of new microarray-based automated techniques

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allowing for parallel analysis of multiple DNA samples. Today, two major applications of DNA microarrays are gene expression profiling and gene mutation analysis (Golub et al., 1999; Tavazoie et al., 1999; Brenton et al., 2001; Hoheisel, 2006).

The application of DNA microarrays in mutation studies is still at an early stage, although they are from a conceptual point of view much simpler. Among the genomic mutations, single nucleotide polymorphisms (SNPs) are the most suitable targets for DNA microarray analysis, whereas multiple mutations, insertions, deletions and rearrangements are more challenging (Hoheisel, 2006). However, even SNPs are usually detected by microarrays with relatively poor accuracy. Therefore, an independent verification of new sequence variants is necessary (Hoheisel, 2006; Cargill et al., 1999). Another problem of the use of DNA microarrays for mutation analysis is their high cost and therefore limited availability. Inexpensive DNA microarrays with a flexible design would be helpful for robust and high-throughput mutation analysis. Key issues for such devices are the specificity, sensitivity and the reproducibility of the analytical process particularly.

Conventional substrates used for DNA microarrays are based on glass, silicon or evaporated gold (on glass). The implementation of these substrates in biochip production requires modification by surface-attached monolayers, frequently called self-assembled monolayers, to which the DNA oligomer is coupled in a subsequent reaction step (Schena et al., 1995; The Chipping Forecast I, 1999; The Chipping Forecast II, 2002). Examples for such processes are binding of thiol-modified oligonucleotides to silicon wafers coated with alkyltrichlorosilanes (Wulfkuhle et al., 2003), binding of 5′-end amino modified oligonucleotides to 3-glycidoxypropyltrimethoxy-silane coated planar waveguides (Shoemaker et al., 2001), tethering of DNA to glass slides by an epoxysilane-amine covalent linkage (Scherf et al., 2000), as well as coupling of oligonucleotides to a thiol SAM on gold (Nuwaysir et al., 1999). However, for immobilisation of DNA, some of these reactive monolayers require a detailed strategy to prevent unspecific binding, i.e. by specific blocking procedures. If thiol or sulphide self-assembled monolayers on gold are employed, the layers are not thermally stable and, if exposed to air for longer periods of time, may oxidise (Jeffreys et al., 1985).

Toomey et al. (2004) reported on the covalent attachment of a polymer film through a photochemical process. UV-illumination crosslinks the polymer molecules and simultaneously links them to the surface, forming stable surface-attached networks that do not delaminate from the substrate upon swelling. The degree of swelling of these networks can be controlled by the content of the photo-reactive groups in the polymer.

In this work results of experiments are presented, which use this procedure to generate DNA biochips for genotyping HPV. A standardised protocol for a labelling PCR was established for target specific amplification of the late 1 region (L1) of the HPV genome. By amplicon hybridisation this new polymer-based DNA biochip platform is used to analyze paraffin-embedded biopsy slices. The specificity, sensitivity and robustness of the obtained biochips are elucidated.

2. Materials and methods

2.1. Generation of the polymers

Poly(dimethylacrylamide-co-(4-methacryloyloxy benzophenone)-co(vinyl phosphonic acid)) was synthesised from the respective monomers by free radical polymerisation. Dimethylacrylamide (DMAA) and vinyl phosphonic acid (VPA) were purchased from Sigma–Aldrich and Fluka. 4-MABP was synthesised as described by Toomey et al. (2004). Polymerisation was carried out in toluene at 60 °C using 1 mol% azobis-isobutyronitril (AIBN, Fluka) as initiator. After completion of the polymerisation reaction, the polymer was precipitated with 10× (v/v) diethylether. The precipitate was isolated and redissolved in water (10 mg/ml). For further purification the polymer solution was dialyzed in water for 7 days. Finally, the polymer was freeze-dried and stored in a light-protected flask at 4 °C.

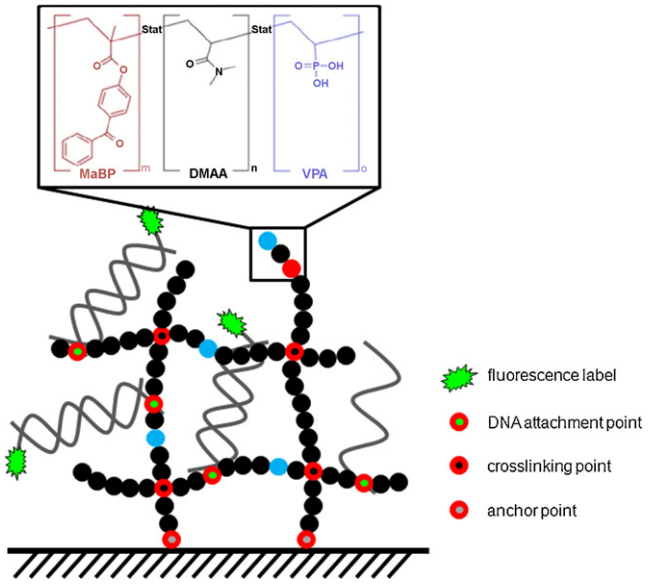

Fig. 1. Schematic depiction of the structure of the nodes of the DNA microarray together with the chemical composition of the polymer used for the generation of the hydrogel network. The photoactive benzophenone units are used in a photoreaction step to simultaneously crosslink the polymer, bind the DNA and attach the forming, functionalised gel to the surface. The fluorescent labelled PCR amplicon hybridise to the immobilised probes.

The composition of the polymer (Fig. 1) was determined using NMR (nuclear magnetic resonance), GPC (gel permeation chromatography) and titration analysis.

2.2. Printing of the DNA arrays

63 probe-oligo sequences with a 5′-modification in form of a 15 mer thymine tail (TIBMOLBIOL, Berlin; HPLC purified) were printed in four equal sub-arrays (2 × 2) using the *sciFLEXARRAYER* S5 (scienion AG). The oligo probes were spotted at a concentration of 20 μM in a spotting solution consisting of a 400 mM sodium phosphate buffer, pH 7.0 (Napi buffer) on commercially available surfaces (polymethylmethacrylate [PMMA]). The spotting solution also contained 1 mg/ml polymer. The printing temperature was 22 °C and the humidity 60–65%. The spot volume was about 0.8 nl per dot resulting in a spot diameter of about 175 μm. The spot spacing was chosen as 350 μm and spacing of the sub-arrays was 500 μm. The printed arrays were annealed for 1 h at 70 °C, followed by UV-irradiation at 254 nm with 1.25 J in a stratalinker (Stratagene Europe). After printing the slides were stored at room temperature. The sub-array design is shown in Fig. 2. Three oligo probes target one HPV genotype. The HPV PCR product of about 405 bp consists of three heterogeneous domains in the L1-region of the HPV genome. The exact length depends on the HPV genotype. 12 HPV genotypes were selected for the layout of the chip. Included in the arrays were a negative control (NC), which contained the complete spotting solution without DNA, which allows background determination, one negative control containing a sequence from the human leukocyte antigen gene DRB1 (NCHL), one detection control containing a 3′-biotinylated oligo probe (biotin, DC), serially diluted hybridisation control (HC) as HPV positive control, which consisted of a universal antisense HPV sequence from the L1-region; a line of